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Pole Cells of *Drosophila paulistorum*: Embryologic Differentiation with Symbionts*

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Abstract

The pole cells of young *D. paulistorum* embryos are destined to form the germinal cells of both male and female imagoes. In addition, specialized portions of the midgut may be derived from pole cell progenitors. In this initial study of their embryogenesis by means of electron microscopy, various stages of pole cell development are shown in both non-hybrid (potentially fertile) and intersemispecific hybrid (potentially sterile as males) materials. Originally, approximately 5 or 6 cells emerge to form the early polar cap and subsequently divide asynchronously until the 35–50 cells of the late polar cap are derived. Unlike other *Drosophila* species, however, mycoplasma-like symbionts, apparently an hereditary infection, have been traced to locations within the cytoplasm of these pole cells. They are depicted as arriving there after transmission via the egg cytoplasm, implicating this as their probable route of entry into the future germinal tissues of adult flies. It is postulated that these microorganisms function as an infectious reproductive isolating mechanism fostering hybrid male sterility between *D. paulistorum* semispecies.

Introduction

King (1972) defined the pole cell as one of the cells that is precociously segregated into the posterior pole of the insect embryo before blastoderm formation; among these cells are the progenitors of the germ cells. The posterior pole of the dipteran egg was first cited as the location of cells serving as a subsequent source of gametes by Mecznikoff in 1865. In non-dipteran insects this was verified experimentally by Hegner (1909). He mutilated beetle eggs so that material from the posterior pole was partly or wholly lost, and raised adults without germ cells (Hegner 1908, 1911, 1914). Later, fertilized *D. melanogaster* eggs were irradiated with ultraviolet light (Geigy 1931) and subjected to ultrasonic sound (Counce and Selman 1955); this resulted in the destruction of the by-then-named pole cells and the production of sterile adults. In 1964, Jura considered the origin, fate and effects of treatment of pole cells in *D. virilis*. Finally, Poulson and Waterhouse (1960) established the essential role of the pole cells in Diptera with respect to the development of a fertile imago in these holometabolous insects. They also implicated pole cells in the differentiation of specialized portions of the midgut, a site of pole cell migration. Engelmann (1970) reviewed the evidence historically, reminding his readers that *Drosophila* spp., which do not produce sex hormones, have the usually assigned function of sex hormones performed by their pole cells. These cells are neither ecto-, meso- nor endodermal in origin but are totally unique.

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The species-complex *D. paulistorum* is also unique: it is composed of six or more semispecies, neotropical in distribution. Dobzhansky (1970, and references therein), Dobzhansky and Pavlovsky (1971) and collaborators should be consulted for charts of the geography and crossability of this cluster of reproductively isolated populations. The primary extrinsic mechanism differentiating the *D. paulistorum* semispecies is geographic isolation, albeit incompletely because of consistently overlapping distributions. (We know almost nothing about the insects' ecology.) Two intrinsic isolating mechanisms are operative in this material, sexual (or behavioural or ethologic) isolation and hybrid male sterility (see Dobzhansky and Pavlovsky 1971).

The factors responsible for the intersemispecific hybrid male sterility within the superspecies *D. paulistorum* are unique in that they can be transmitted by injection, i.e. are infectious, and can be partially, temporarily suppressed by heat shocks or antibiotics or both (Ehrman 1967, 1968). Ultimately, however, the sterility of the hybrid F_1 or backcross male depends upon the genotype of its mother (Ehrman 1960; Perez-Salas and Ehrman 1971).

Materials and Methods

Crosses in either direction between the Mesitas, Colombia, Andean–Brazilian semispecies and the Santa Marta, Colombia, Transitional semispecies, both from South America, produce fertile daughters and sterile sons, when sexual isolation is overcome and such crosses succeed. These hybrids are forced into existence in the laboratory; they have not been collected thus far in nature. Dobzhansky and Spassky (1959), Ehrman (1963), and Ayala *et al.* (1970) may be consulted for details of the origins and histories of these and of other *D. paulistorum* strains. The embryos studied here were produced by (Mesitas female × Santa Marta male) F_1 hybrid females who had been inseminated by Santa Marta males (see Ehrman 1963, fig. 12). Adult males derived from such crosses are sterile.

We have previously briefly itemized the techniques employed (Daniels and Ehrman 1974); in detail, we relied upon the recommendation of Farnsworth (1963), Mahowald (1968), Hayat (1970), and Kalt and Tandler (1971). Timed embryos were harvested at age 1·5–2·0 h by observing the oviposition of fertilized eggs by gravid females, and were then washed in physiologic insect saline or in distilled water to remove any adhering food. The chorions were removed mechanically following Farnsworth's (1963) suggestions: eggs were transferred in a medicine dropper onto the adhesive side of Scotch tape fastened to a glass slide and excess water or saline was drawn off with filter paper, causing the chorion to adhere to the tape. By gently stroking the egg with a fine brush, or by prodding it with a blunt needle, the chorion was ruptured and its removal from the embryo facilitated. Although this method makes the surface of the vitelline membrane hydrophobic and the process of fixation slightly more difficult, the alternative of dechorionating embryos with a solution of sodium hypochlorite was avoided because of possible induction of ultrastructural artifacts in the pole cells.

Dechorionated embryos were placed on a microscope slide with a coverslip and observed under phase contrast microscopy at magnifications of 80–120. Those embryos displaying stages of polar cap formation were placed in fixative at room temperature and punctured with a tungsten needle sharpened in molten sodium nitrite. After the fixative had been allowed to penetrate for 20 min, the vitelline membrane was carefully dissected from the embryo. This step is essential to allow for adequate penetration of the fixative and to eliminate tissue compression during dehydration due to contraction of an intact vitelline membrane (Mahowald 1968).

Standard fixation techniques employed by previous investigators did not yield high quality preservation of early *Drosophila* embryos, probably because of their hydrated state and cytoplasmic fragility (Mahowald 1968). In order to remedy this situation, a trialdehyde fixative was initially used for this study. Pure Mesitas embryos were fixed in a mixture of 3% glutaraldehyde, 1·5% paraformaldehyde and 1% acrolein in 0·1M phosphate buffer at pH 7·6 for 1·5–2·0 h at room temperature. Embryos were then rinsed three times in buffer, post-fixed in 1% OsO_4 in 0·1M phosphate buffer, dehydrated in a graded ethanol series and embedded in Epon 812.

With this fixation technique certain previously reported artifacts are avoided, e.g. disruption of the cytoplasmic matrix and distention of membranes, but other problems arise. The cytoplasm appears granular and membranes, although undilated, are not well preserved. This is especially evident in mitochondria, where integrity is maintained but limiting membranes are ill-defined. Nuclear membranes appear uninterrupted and evenly spaced, but also show this same, somewhat inadequate degree of preservation. A fixation procedure recently described by Kalt and Tandler (1971) as providing exceptional preservation of embryonic tissue was then employed on our hybrid embryos.

Initial fixation took place in a trialdehyde–DMSO (dimethyl sulphoxide) mixture consisting of 3% glutaraldehyde, 2% paraformaldehyde, 1% acrolein, 2·5% DMSO in 0·1M cacodylate buffer (containing 0·001M $CaCl_2$) at pH 7·4 for 12–16 h. Primary fixation was carried out at room temperature for 1·0–2·0 h, at which time the temperature was reduced to 4°C. All subsequent steps were executed at this temperature until the one utilizing 70% acetone was reached. After primary fixation, embryos were washed at 15-min intervals in 0·1M buffer in 1·0M sucrose for 60–90 min, post-fixed in a solution containing 2% osmium tetroxide, 0·2M sucrose and 0·1M cacodylate buffer for 6–8 h, and then rinsed in distilled water. With embryonic tissues, the length of post-fixation time in OsO_4 is a crucial factor in membrane preservation; prolonged fixation seems to be essential if membranes are to be adequately preserved. Embryos were then stained *en bloc* in 0·5% aqueous uranyl acetate for at least 2 h and dehydrated in a graded acetone series culminating in propylene oxide and followed by prolonged infiltration with Epon 812. Acetone dehydration and prolonged infiltration of embedding media are recommended because such processes aid in reducing the heterogeneity of early embryologic tissue produced primarily by yolk platelets (Kalt and Tandler 1971).

Sections were mounted on Formvar-coated or Formvar carbon-coated 100-mesh grids, stained with lead citrate (Reynolds 1963), and optionally with uranyl acetate (Hayat 1970 recommends staining with uranyl acetate even if the tissue previously has been stained *en bloc*). Observations were made with a JEM T6S electron microscope.

Observations and Discussion

Pole cell formation along with vitellophage differentiation constitute what appears to be the first cellular differentiation of a developing *Drosophila* embryo (Sonnenblick 1950; Counce and Waddington 1972, 1973). These cells form when three or more cleavage nuclei migrate to the posterior cytoplasmic evaginations which then pinch off from the ooplasm (see Figs 1–3). This results in the precocious segregation of pole cells from the rest of the developing embryo. Usually five or six cells emerge to form the initial polar cap, although this number may vary from as few as three to as many as eight or more. Once the initial polar cap is formed, no new cells enter the cap from the ooplasm. Pole cell proliferation continues by way of asynchronous mitotic divisions (Fig. 6) until 35–50 cells are present in the mature polar cap. The nuclei of the blastema or acellular blastoderm continue to divide three more times after initial pole cell formation, completing a total of 11 divisions (Mahowald 1968). The mature polar cap is swept into the developing posterior midgut invagination 60–75 min after its initial formation (Anderson 1972), and is carried into the interior of the embryo as the lumen deepens (Fig. 7). For ultrastructural comparison of hybrid and non-hybrid embryos, see Figs 4 and 5.

The most outstanding characteristic of any pole cell is the presence of polar granules within its cytoplasm (Figs 4–6). It has been hypothesized that the RNA present in these polar granules is mRNA which directs the synthesis of proteins necessary for the transformation of pole cells into germinal ones (Mahowald 1968, 1971*a*, 1971*b*). Polar granules of *D. paulistorum* are characteristically doughnut-shaped; a closely related neotropical sibling species, *D. willistoni*, has polar granules of similar morphology (Counce 1963; Mahowald 1968).

Under light microscopy, Mesitas–Santa Marta hybrid embryos frequently display gross abnormalities in development. These abnormalities usually occur around the time of presumptive pole cell differentiation. Observed deviations include the formation of abnormally large pole cells, pole cells emerging along abnormal egg (ventral or dorsal) surfaces instead of at the posterior tip, large numbers of emerging pole cells, blastoderm formation without requisite prior pole cell differentiation, and assorted other chronological errors of differentiation. These aberrations often lead to an arrest in development and the gradual disintegration of the entire embryo.

Hybrid eggs (unusually adherent to our instruments) differ in many gross respects from non-hybrid ones produced by pure *D. paulistorum* semispecies. They frequently lack the turgidity, uniformity of shape and cytoplasmic consistency characteristic of a healthy non-hybrid egg. We measured 20 hybrid eggs and calculated an average length of 520 μm for them (from micropyle to posterior end, exclusive of filament length); for 20 non-hybrid Mesitas eggs, the equivalent average was 495 μm.

On the ultrastructural level, pleomorphic symbionts previously identified as mycoplasma-like microorganisms (MLO)* were observed in forming and in fully differentiated pole cells of both hybrid and non-hybrid embryos (Figs 6–13). These intracellular microorganisms were always observed to possess two and sometimes three or four enveloping membranes. Internally, MLO are typically characterized by electron dense ribosome-like particles and indications of fibrillar strands of condensed DNA (Figs 11–13).

Three interrelated kinds of male sterility occur in hybrids between semispecies of the *D. paulistorum* complex (Williamson *et al.* 1971 and references therein). F_1 hybrid males are always sterile because of their own genetic constitution; some or all backcross hybrid males are sterile because of their mothers' genotypes; the third type of sterility is due to a maternal effect: there is an interaction between an omnipresent cytoplasmic symbiont and the mother's genotype. This last type, of interest here, is best exemplified by supposing that two strains representing different *D. paulistorum* semispecies, denoted A and B, produce sterile male hybrids when crossed. If sterile A/B hybrid males are ground, suspensions centrifuged, and the supernatant injected into virgin B females which are then crossed to B males, non-hybrid but sterile B sons are subsequently produced.

The target organ system in all these types of sterility is the male reproductive tract, and it has been shown to harbour MLO which seem to exist in greater numbers in the testes of sterile males than they do in the testes of fertile ones. In aged sterile males, MLO eventually occupy entire testicular sacs, no cell in any stage of spermatogenesis being present. These MLO have been implicated in, but not proven to be the cause of, the hybrid male sterility in this species-complex (Ehrman and Kernaghan 1972 and references therein), and to be under the control of nuclear

* According to the recommendation of Freundt (1973), we use the informal term 'mycoplasma-like' in its broadest sense, to encompass any member of the class Mollicutes, including two genera, *Mycoplasma* and *Acholeplasma*.

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nous mitotic divisions. Note the characteristic difference in cytoplasmic density between the pole cell and the egg cytoplasm. This difference is mainly due to a greater concentration of ribosomes and polysomes in the latter. In this micrograph the blastema is in late prophase, as indicated by one of its nuclei (arrow) in the posterior cytoplasm below the fully formed pole cells. *PG*, polar granules.

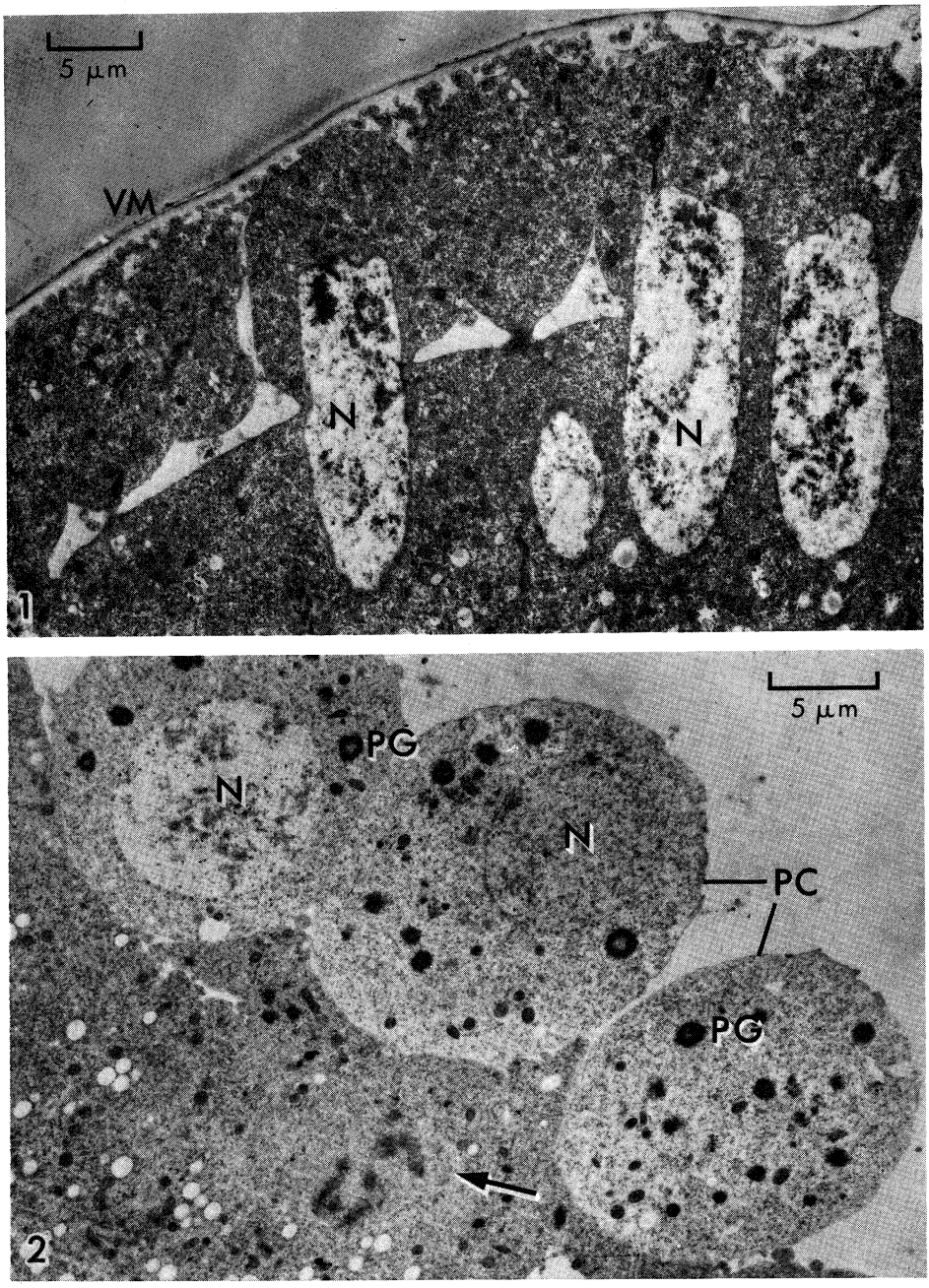


Fig. 1. Formation of pole cells from the posterior tip of a (Mesitas ♀ × Santa Marta ♂) F_1 ♀ × Santa Marta ♂ hybrid embryo. Nuclei (*N*) which have migrated into the posterior cytoplasm are seen moving into cytoplasmic protuberances which will eventually pinch off to form pole cells. The vitelline membrane (*VM*) lies just beneath the chorion of the egg; it is covered on the exterior by a waxy substance which is laid down on the membrane during the last stages of oogenesis. This waxy material is seen as a darkened border along the membrane surface.

Fig. 2. Portion of a newly formed polar cap of a non-hybrid Mesitas embryo. The pole cells (*PC*) have just recently pinched off from the rest of the ooplasm and have not as yet undergone asynchro-

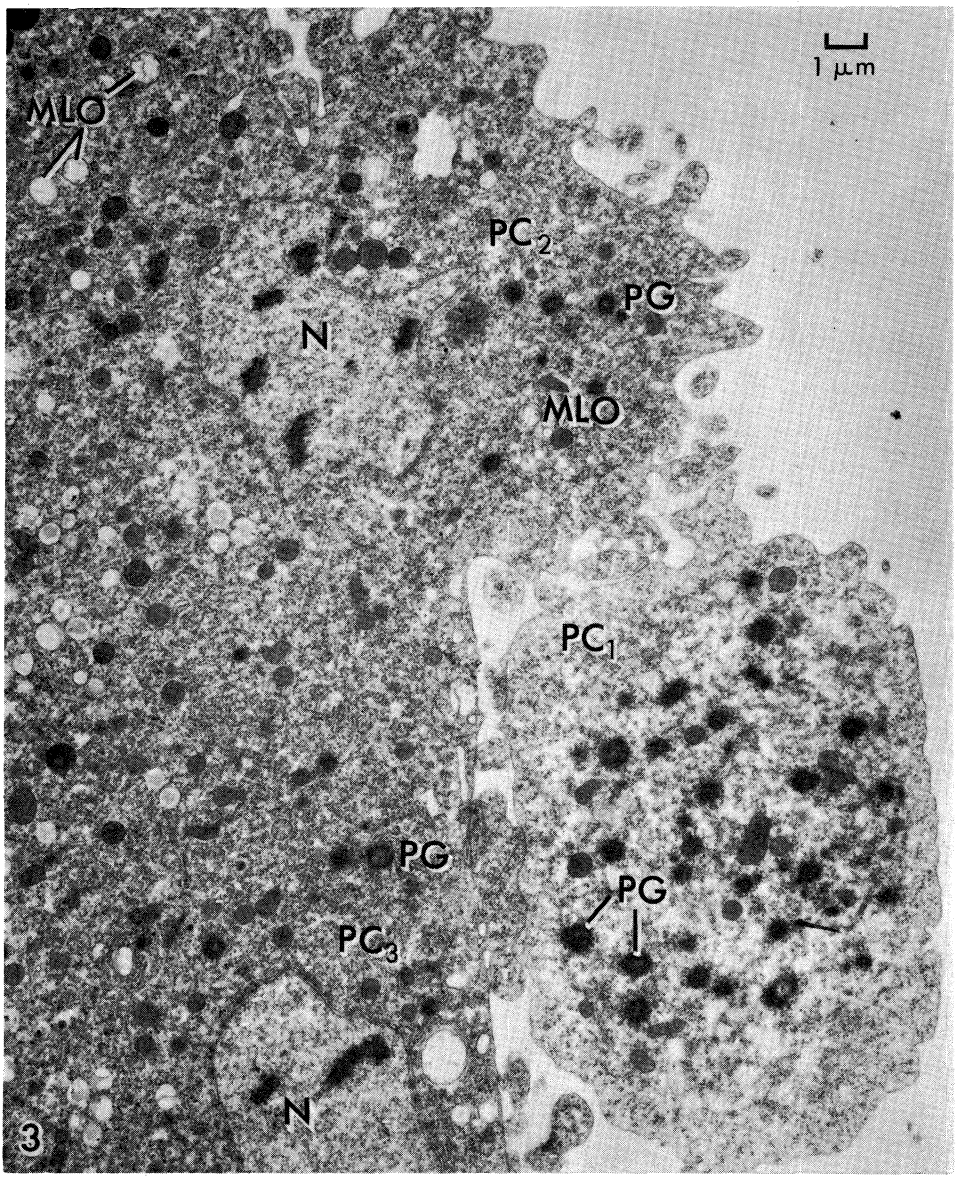
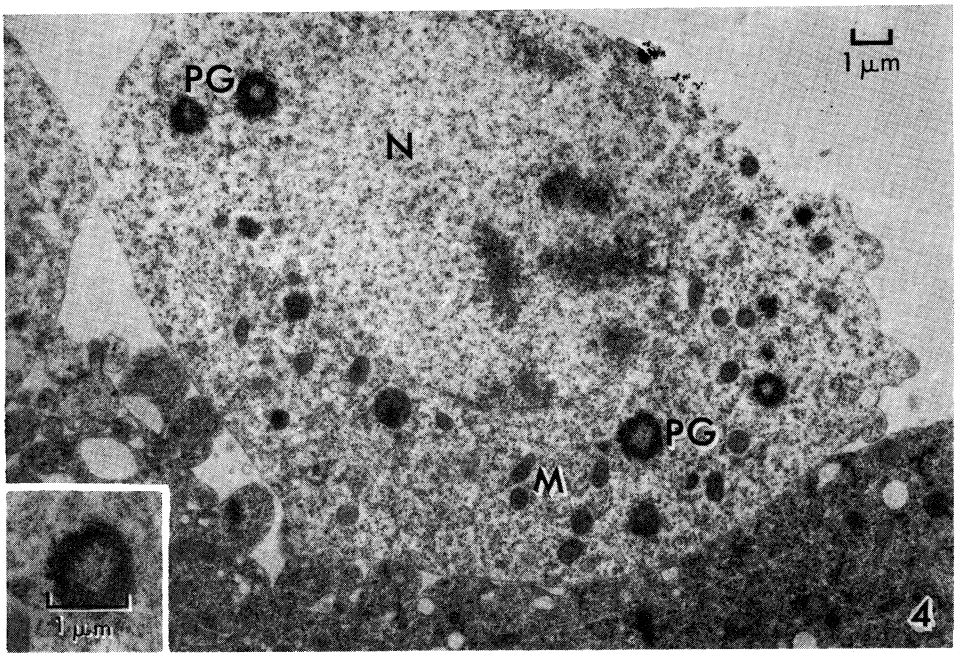
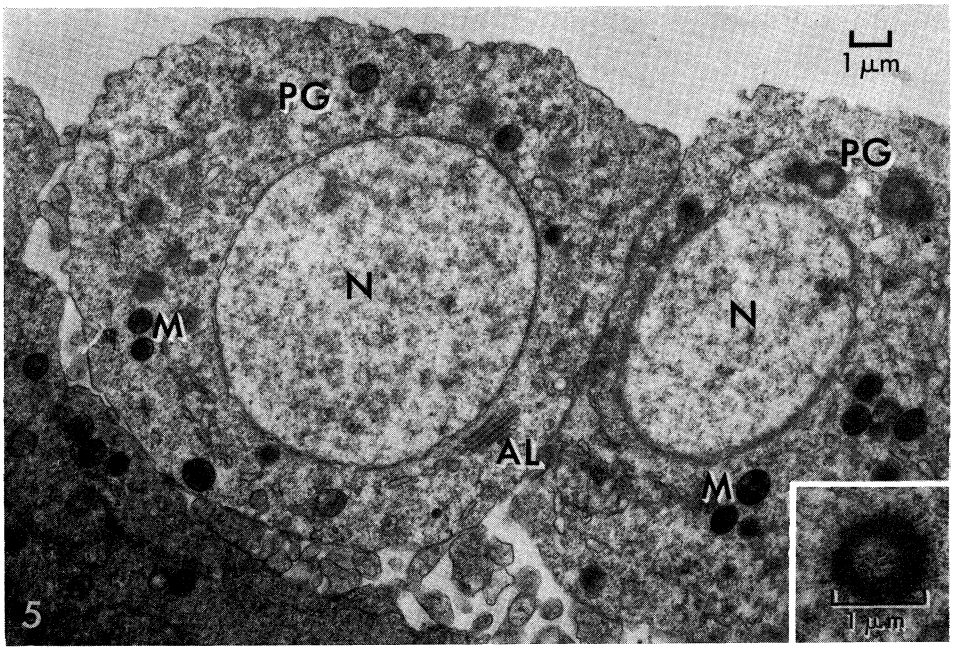
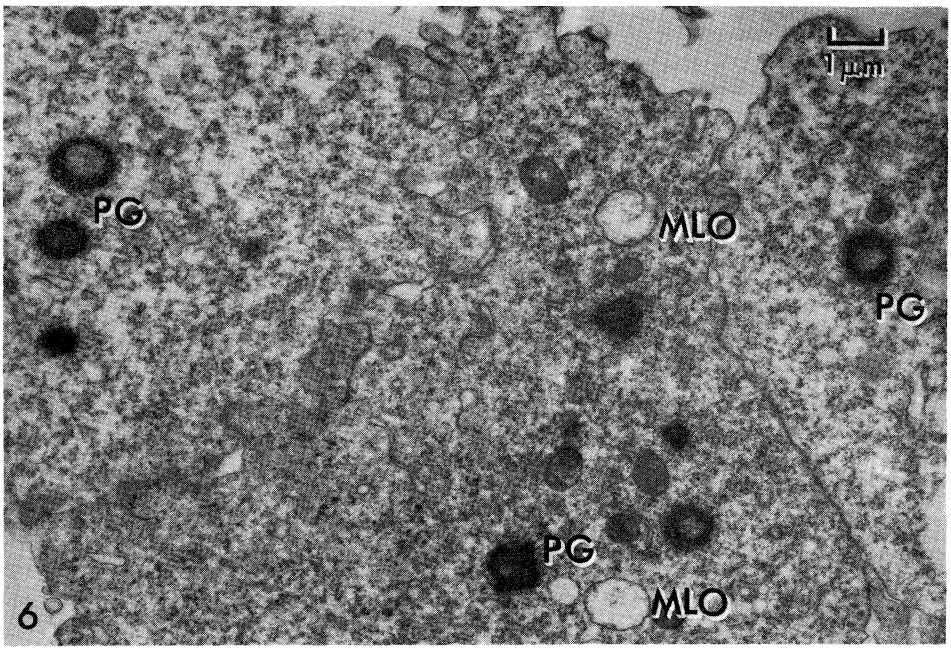


Fig. 3. Three hybrid pole cells in different stages of formation. PC_1 is a fully formed pole cell; PC_2 depicts the cytoplasmic protuberance into which the migrating nucleus moves; and putative PC_3 as yet has no cellular boundaries; PC_3, whose future as either a pole cell or a blastoderm cell cannot at this point be definitely stated, may be considered an incipient pole cell because of the presence of polar granules (*PG*) in the posterior cytoplasm surrounding its nucleus. Note that mycoplasma-like organisms (*MLO*) can be seen in the cytoplasmic protuberance as well as scattered throughout the posterior cytoplasm. These MLO may be incorporated within pole cells as formation proceeds and cell boundaries are established.





Figs 4 and 5. Comparison of non-hybrid (Fig. 4) and hybrid (Fig. 5) pole cells. It is evident that there are basically no ultrastructural differences which distinguish hybrid from non-hybrid cells. However, certain hybrid embryos that develop abnormally often display grossly aberrant pole cell formation (see text). Typically, pole cells are characterized by ovoid nuclei (*N*), polar granules (*PG*), frequently annulate lamellae (*AL*), free polyribosomes in the cytoplasm, and other cell organelles, e.g. mitochondria (*M*), endoplasmic reticulum and Golgi complexes. Note that the results of the Kalt and Tandler fixation procedure (Fig. 5) are superior to those obtained with the initial trialdehyde fixation process (Fig. 4).



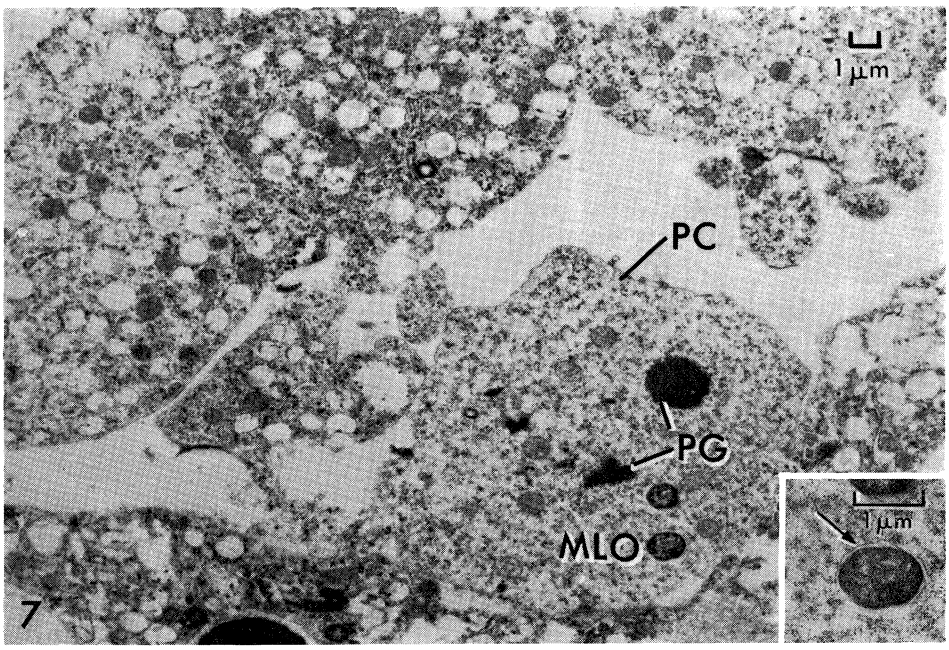


Fig. 6. Hybrid pole cells in late telophase. Cell boundaries are being established. MLO are seen in all stages of pole cell development.

Fig. 7. A hybrid pole cell (*PC*) in the posterior midgut rudiment. As pole cells pass through developmental stages, MLO within them begin to display more internal structure and more well-defined limiting membranes (see arrow in inset).

genes (Perez-Salas and Ehrman 1971). In this unique instance, then, they apparently serve as an infectious microbial reproductive isolating mechanism.

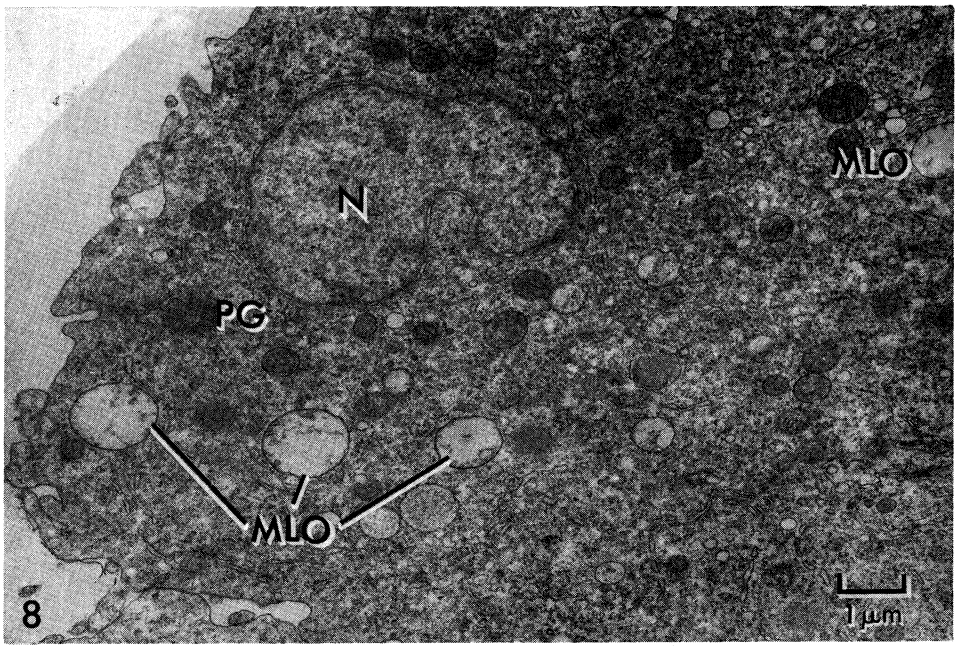


Fig. 8. A forming hybrid pole cell showing MLO within cytoplasmic evagination. These MLO are captured as pole cell formation is completed and cell boundaries are established. *PG*, polar granules; *N*, nucleus.

The transfer of MLO vertically, between generations, occurs via the egg cytoplasm. Several hundred MLO, as crudely estimated by Kernaghan from electron micrographs, may be scattered throughout the cytoplasm of a single egg. After fertilization and the initiation of many nuclear divisions, such symbionts are dispersed and captured as cell boundaries are established (Fig. 8). The early segregation of those cells destined to form the rudimentary larval germinal tissue (pole cells) ensures the transmission of these symbionts to adult reproductive organs, where they are omnipresent (Figs 9 and 10). No untreated *D. paulistorum*, male or female, hybrid or non-hybrid, have ever been observed to be free of MLO, though only a limited number can and have been surveyed with an electron microscope. Mahowald has located symbionts in *D. willistoni*, a closely related species (see Mahowald 1968, fig. 8; 1971*a*, fig. 4).

We have previously (Kernaghan and Ehrman 1970; Ehrman and Kernaghan 1971) documented the presence of intracellular microorganisms in the cytoplasm of cells of the adult ovaries and testes (including spermatids) of hybrid as well as non-hybrid *D. paulistorum* adults. More recently, Kernaghan presented ultrastructural evidence of the presence of MLO within the cytoplasm of cells of the third instar larval gonadal primordia of both sexes (Ehrman and Kernaghan 1972). These same intracellular entities have also been seen in considerable numbers in germinal follicle and nutritive cells of the ovary, as well as in unfertilized and fertilized eggs; follicle

cells, for instance, are of mesodermal origin, and therefore could represent an additional source of infection.

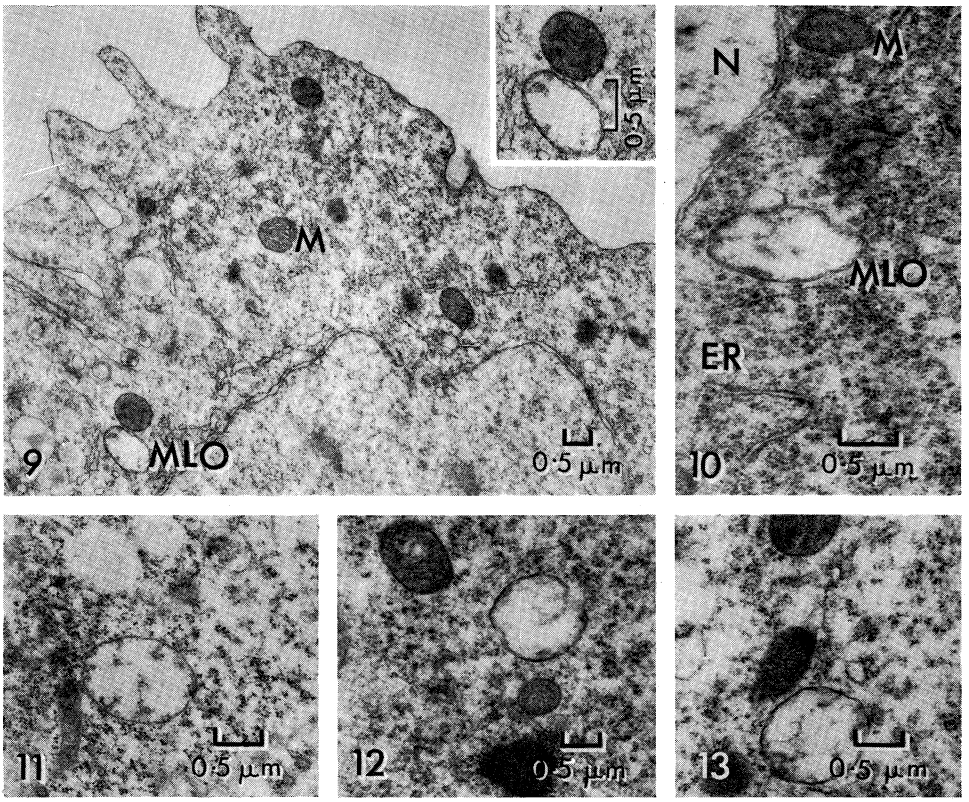


Fig. 9. Distal portion of a newly formed hybrid pole cell with an accompanying MLO in the perinuclear space. *M*, mitochondrion.

Fig. 10. An MLO in the perinuclear space of a hybrid pole cell. *ER*, endoplasmic reticulum.

Figs 11–13. MLO in the cytoplasm of pole cells. Fig. 11 shows an MLO in a non-hybrid Mesitas pole cell; Figs 12 and 13 depict MLO within hybrid pole cells.

With this current demonstration of the presence of MLO within pole cells, they have now been traced throughout their host's life cycle. These important cells are destined, after being carried into the posterior midgut rudiment, to form either female or male germ cells (Mahowald 1971*c*). Since our mycoplasma-like symbiont resides only intracytoplasmically, and the cytoplasm of spermatozoa is virtually or actually eliminated with mature functioning, it is by means of the egg cytoplasm that transmission occurs between generations. Although the act of transmission resides with females, the ultimate result of this transmission is evident in the sterile testes of *D. paulistorum* hybrid males. We note here the impressive new, eminently applicable, technique described by Okada *et al.* (1974*a*, 1974*b*), involving the transfer by injection of polar cytoplasm. We have, as yet, been unable to grow the *D. paulistorum* MLO *in vitro* (Ehrman and Ramos 1973).

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